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Method of chloroplast transformation in asteraceae

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METHOD OF CHLOROPLAST TRANSFORMATION IN *ASTERACEAE*

Field of the invention

The invention relates to methods of genetically transforming plant plastids, and more specifically to genetically transforming the plastid genomes of *Asteraceae* plant species.

Background

Plant plastids (e.g. chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts) are organelles in which major biochemical processes (i.e. photosynthesis) take place. In general, plant cells contain between 100-10,000 copies of a small 120-160 kb circular genome. Since each molecule has one inverted repeat it is theoretically possible to obtain plant cells with 20,000 copies of (a) gene(s) of interest, after chloroplast transformation.

The genetic transformation of the plastid genome (plastome) has major advantages over nuclear transformation. Firstly, in most plant species, plastids are maternally inherited, which minimizes out-crossing of transgenes related to weeds or other crops. This form of genetic engineering of plants lowers the risk of dissemination of the transgene in the environment through pollen dispersal. Furthermore, the plastid genome is highly polyploid, enabling the introduction of many copies per cell which can lead to high accumulation levels of the desired protein(s). The fact that chloroplasts are able to form disulfide bands and to fold human proteins, makes this technique ready for the production of biopharmaceuticals in plants.

The principle of chloroplast transformation is transfer of sequences through homologous recombination. Chloroplast transformation vectors use two targeting vectors

that flank the foreign genes and insert them, at a precise, predetermined position in the chloroplast genome. Position effects and gene silencing, major problems in nuclear transformation experiments, have not as yet been observed in
5 chloroplast transformation events.

Successful chloroplast transformation of crop plants is described thus far only for *Solanaceous* crops like potato, tomato, tobacco (U.S. Patent Number 5.451,513; Svab et al. (1990), *Proc. Natl Acad. Sci USA* 87:8526-8530) and rapeseed.

10

Detailed description of the invention

Methods and compositions for an efficient and stable transformation of chloroplasts of a lettuce plant are provided. DNA constructs are provided for stable
15 transformation of plastids of multicellular structures and plants of lettuce.

As explant material, plant mesophyll protoplasts are used and via PEG transformation transplastomic protoplast-derived colonies and regeneration of meristems were obtained.
20 The DNA constructs comprise a transforming DNA which is targeted to a pre-determined location in the plastid genome and inserted into the plastid genome by homologous recombination. The targeting segments comprise preferred sequences of the lettuce DNA chloroplast genome, i.e. the
25 *trnI(oriA)/trnA* region or the 16S/*trnV*/ORF70B region of the lettuce chloroplast genome. The DNA used for transformation contains a non-lethal selectable marker gene which confers a selectable phenotype on cells having the plastids with the transforming DNA. Furthermore the transforming DNA comprises
30 at least one additional DNA sequence, such as encoding a green fluorescent protein (gfp). The non-selectable marker coding segment preferred, is the coding region of *aadA* from bacteria (*E. coli*) which encodes aminoglycoside 3 "-

adenylyltransferase to confer spectinomycin and streptomycin resistance. The constructs furthermore are provided with a promoter and a terminator sequence functional in plant plastids.

5

Legend to Figure 1:

A: PCR products of the ATPase gene.

1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
- 10 3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
- 15 8. TRSL5-02002 pLCV2-MSK18-2-2,
- 9 and 10 untransformed callus,
- 11 and 12 pLCV2-MSK18

B: PCR products of the AadA gene.

1. Marker,
- 20 2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
- 25 7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
- 9 and 10 untransformed callus,
- 11 and 12 pLCV2-MSK18

C: PCR products of the trnI junction.

- 30 1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,

5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
- 9 untransformed callus

D: PCR products of the trnA junction.

1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
- 10 4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
- 15 9 untransformed callus

EXAMPLES

EXAMPLE 1

20 Vector constructions

Construction of LCV1

The lettuce chloroplast vector LCV1 consists of 4571 bp of lettuce chloroplast genome sequence with a unique 16 bp *PacI/AscI* site added (Figure 1), cloned into *SacI/KpnI* restriction sites on the polylinker of a pBluescript SK+ backbone vector (Figure 2). The lettuce sequence spans from the *rps7/3'-rps12* intergenic region to the *16S rRNA/trnI* intergenic region and corresponds to nucleotide positions 100021-104387 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in Figure 3. The following description of the construction of LCV1 is outlined in Figure 4.

Four primers LCV1A, LCV1B, LCV1C and LCV1D were used to amplify this region in two halves (LCV1A-B and LCV1C-D) and to introduce a unique *PacI*/*AscI* restriction site in the ORF70B/*trnV* intergenic region at the position corresponding to nt 102367 in the tobacco chloroplast genome sequence. DNA from clone 6 of the *SacI* library of the lettuce chloroplast genome (Jansen and Palmer, 1987) was used as a template for the LCV1 vector. LCV1A and LCV1B amplified a 2575 bp fragment (2551 bp lettuce sequence + 24 bp extension) LCV1A-B spanning from the *rps7/3'*-*rps12* intergenic to the ORF70B/*trnV* intergenic region (corresponding to 100021-102367 in the tobacco chloroplast genome). Primer LCV1A contains a *SacI* site and LCV1B contains *PacI*/*AscI* sites so that *SacI* and *PacI*/*AscI* sites are incorporated at the 5' and 3' end, respectively, of the LCV1A-B fragment. The LCV1 A-B fragment was cloned into the *E.coli* plasmid vector PCR2.1 to create PCR2.1 LCV1A-B. These clones were screened for orientation using *SacI* and *SacI*+*XbaI*. The *SacI*/*XbaI* insert was cloned into the polylinker of pBluescript to create pBSLCV1 A-B. Primers LCV1C and LCV1D amplified a 2042 bp fragment (2020 bp lettuce sequence + 22 bp extension) LCV1 C-D. The LCV1C primer contains *PacI*/*AscI* sites and the LCV1D primer contains a *KpnI* site so that a *PacI*/*AscI* and a *KpnI* site are added to the 5' and 3' end, respectively, of the LCV1 C-D fragment. The LCV1 C-D fragment was cloned into PCR2.1 to create PCR2.1 LCV1 C-D. For the final cloning step, PCR2.1 LCV1 C-D was restricted with *AscI*+*KpnI* to release a 2031 base pair insert that was ligated to pBS A-B, which was linearised with *AscI*+*KpnI*, creating LCV1.

Construction of LCV2

LCV2 consists of a 2253 bp lettuce chloroplast genome sequence (Figure 5) spanning from the 16S rRNA/*trnI*

intergenic region to the *trnA*/23S rRNA intergenic region, cloned into the PCR2.1 (Invitrogen) backbone vector (Figure 6). This sequence corresponds to nucleotide positions 104366-106260 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in (Figure 7). The following description of the construction of LCV2 is outlined in Figure 8. Four primers LCV2A, LCV2B, LCV2C and LCV2D were used to amplify this region in two halves (LCV2A-B and LCV2C-D) and to introduce unique *PacI*/*AscI* restriction sites in the intergenic region between the *trnI* and *trnA* genes at the position corresponding to nucleotide 105370 in the tobacco chloroplast genome. For the first half (A-B) of the vector, DNA from clone 6 of the *SacI* library of the lettuce chloroplast genome (Jansen and Palmer, 1987) was used as a template. Primers LCV2A and LCV2B amplified a 1258 bp fragment (1242 bp lettuce sequence + 16 bp extension) (LCV2A-B) spanning from 16SrRNA/*trnI* intergenic region to the *trnI*/*trnA* intergenic region. This fragment was cloned into the *E. coli* plasmid cloning vector PCR2.1 (Invitrogen) to create PCR2.1 LCV2A-B. Primer LCV2B contains *PacI*/*AscI* sites so that the LCV2A-B fragment has *PacI*/*AscI* sites at the 3' end. PCR2.1 LCV2 A-B clones were screened for orientation by digestion with *KpnI*/*AscI*, which releases a fragment of approximately 1300 bp, and *XbaI*/*AscI* which linearised clones with the correct orientation for subsequent cloning. For the second half of the vector chloroplast DNA from lettuce cultivar Evola (Leen de Moss seeds) was used as a template because the entire *trnA* gene was not contained in a single clone in the lettuce chloroplast genome library. Primers LCV2C and LCV2D amplified a 1011 bp fragment (995 bp lettuce sequence + 16 bp extension) LCV2C-D. This sequence spans from the *trnI*/*trnA* intergenic region to the *trnA*/23S rRNA

intergenic region. Primer LCV2C contains *PacI*/*AscI* sites so the fragment LCV2C-D has *PacI*/*AscI* sites at its 5' end. This fragment was cloned into PCR2.1 to create PCR2.1 LCV2 C-D. These clones were screened for orientation using *KpnI*+*AscI*, which linearises clones with required orientation and *XbaI*+*AscI*, which releases a fragment of approximately 1000 bp in clones with the required orientation. To generate LCV2, the 1.3 kb *AscI*+*XbaI* insert from PCR2.1 LCV2C-D was subcloned into PCR2.1 LCV2A-B linearised with *AscI*+*XbaI*.

Construction of LCV1-MSK18 and LCV2 MSK18

MSK18 is an expression cassette adapted from pMSK18 (Hibberd et al. 1998). Plasmid MSK18 was a gift from John Gray (Dept. Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK). Full details of the construction of pMSK18 have been described previously (Hibberd et al. 1998). The MSK18 expression cassette consists of the mGFP coding region (Haselhoff et al. 1997) fused to a bacterial *trc* promoter (Amman and Brosius, 1985), and an *aadA* coding region, derived from pUC-atpX-AAD. (Goldschmidt-Clermont, 1991) fused to a tobacco *rrn* promoter derived from pZS197 (Svab and Maliga, 1993). A tobacco *psbA* 3' UTR derived from pSZ197 (Svab and Maliga, 1993) is fused to the 3' end of the *aadA* gene (Figure 9). Using pMSK18 as a template, *PacI* and *AscI* sites were added by PCR amplifying the cassette with primers containing *PacI* (5') and *AscI* (3') restriction sites to 5' and 3' ends of the of the MSK18 expression cassette. The primers used for this were MSK18 A (Forward) 5'-tagttaattaaTTGACAATTAATCATCCGGCTCGT-3' and MSK18 B (Reverse) 5'-tagggcgcgccTCGAATATAGCTCTTCTTTCTTA-3'. The MSK18 A-B PCR product was cloned into PCR2.1 to create PCR2.1 MSK18. PCR2.1 MSK18 was restricted with *PacI*/*AscI* to release the MSK18 insert that was cloned into the *PacI*/*AscI* sites in

LCV1 and LCV2 to create LCV1-MSK18 (Figure 9) and LCV2-MSK18 (Figure 10).

*Molecular characterisation of putative LCV2 transplastomic
5 callus*

Total genomic DNA was extracted from the putatively transformed spectinomycin resistant LCV2-MSK18 callus from seven independent transformation events designated 1, 2, 3, B, D, E and G. PCR analysis of this DNA was used to confirm
10 integration of the LCV2-MSK18 sequence into the lettuce chloroplast genome using a combination of primers (Figure 11). To eliminate the possibility of amplification of unintegrated LCV2-MSK18 plasmid DNA, primers P1 and P4 were designed from lettuce chloroplast sequences external to the
15 vector target region. PCR was carried out on DNA isolated from all 6 putatively transformed calli. In all cases, P1 and P4 give two PCR products, a 2476 bp band corresponding to the expected size of a product amplified from an untransformed wild-type chloroplast genome, and a 4623 bp band
20 corresponding to the size of a PCR product expected from a transformed chloroplast genome (Figure 12). For further confirmation of integration, the left and right integration junctions were amplified by PCR using primer pairs P1+P2 and P3+P4. Primers P2 and P3 correspond to the *trc* promoter and
25 the *aadA* gene on the MSK18 cassette sequence. As expected, PCR amplification with primers P1+P2 and P3+P4 produced products of the expected size (1415 bp and 2006 bp) (Figure 12). The PCR products from callus sample B were cloned into PCR2.1 and sequenced using M13 forward and M13 reverse
30 primers. These sequences confirmed that LCV2-MSK18 was integrated in the lettuce chloroplast genome (Figure 13).

EXAMPLE 2Obtaining seedlings and an in vitro stock of plants

Protoplasts of plants are isolated from leaf material of donor plants. In this example the obtaining of leaf shoot
 5 cultures is given.

Seed is being sterilized by subsequent washing in 70% ethanol, 0.7% NaOCl solution during 20 minutes and three times washing with sterile demineralized water.

Seeds are sown on Murashige and Skoog (Murashige and Skoog,
 10 Physiol. Plant., 15: 473-497, 1962) medium with saccharose 2%, without hormones. Preferentially, seeds can be cultured at 15°C for 2 days in the dark, after which the seeds are transferred to 25°C in the light (3000 lux, photoperiod 16 hr light/8 hr dark). When first true leaves appear, shoot tips
 15 are transferred to Murashige and Skoog based medium with 3% saccharose, without hormones. These sterile shoot cultures are grown under similar growth conditions.

EXAMPLE 3Isolation of protoplasts

Three week old shoot cultures are used for isolation of protoplasts. Leaves are cut into small pieces and preplasmolysed during 1 hr in the dark in PG solution (54.66 g/l sorbitol and 7.35 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). The PG solution is then
 25 replaced by an enzyme solution with 1% cellulase and 0.25% macerozym. Incubation takes place during 16 hrs in the dark at 25°C.

Subsequently, the suspension is filtered through a nylon mesh filter (4 μm) en washed with a third of a volume
 30 of CPW16S solution (Frearson, E.M., Power, J.B. & Cocking, E.S. (1973), Developmental Biology 33:130-137) by centrifugation at 700 rpm during 8 minutes. In this way, intact protoplasts are collected on the surface of the

supernatant. Protoplasts are washed in W5 solution (9 g/l NaCl, 18.38 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.37 g/l KCL, 0.99 g/l glucose, 0.1 g/l Morpholinoethanesulfonide buffer) by centrifugation at 600 rpm during 5 minutes. With the procedure described, a
5 protoplast yield of approximately $10\text{--}15 \times 10^6$ protoplasts per gram leaf material.

EXAMPLE 4

Selection of protoplast derived calli on spectinomycin

10 resistance

Protoplasts of lettuce, derived as described in example 3, are diluted in culture medium $\frac{1}{2}$ B5 (Gamborg et al. (1968) Exp. Cell Res. 50:151): 375 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium succinate, 103 g/l saccharose, 0.1
15 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzylaminopurin (BAP) and set to a culture density of 6×10^4 protoplasts per ml.

The protoplast suspension is mixed 1:1 with $\frac{1}{2}$ B5 culture medium with agarose. The agarose beads are plated in
20 larger petridishes with liquid $\frac{1}{2}$ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C. One week after initiation of culture the culture medium is diluted with fresh liquid $\frac{1}{2}$ B5 culture
25 medium.

When calli are about 0.5 mm in size they are transferred to callusgrowth medium SH2 (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 30 g/l saccharose, 5 g/l agarose, 0.1 mg/l 1-naphtalene acetic acid
30 (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations of 0-1000 mg/l. It was found that the optimal concentration of selection is 500 mg/l. The non-resistant calli will appear as

white calli. They also will grow slower as compared to the control calli.

EXAMPLE 5

- 5 Transformation of protoplasts with polyethylene glycol and selection for aadA encoded antibiotic resistance

Protoplasts of lettuce, derived as described in example , are set to a density of approximately $1-1.5 \times 10^6$ protoplasts/0.4-0.6 ml in transformation buffer (0.4 M mannitol, 15 mM $MgCl_2$, 1% (w/v) Morpholinoethane sulfonate (MES), pH 5.8). Subsequently, 10 μ l of plasmid suspension (1 μ g DNA/ μ l sterile H_2O) is added to the protoplasts as well as 0.4-0.6 ml PEG solution (40% w/v PEG 6000, 2.36 g/l $Ca(NO_3)_2 \cdot 4H_2O$ and 7.28 g/l mannitol). Incubation is performed at room temperature for 5-30 minutes. Protoplasts are washed and resuspended in culture medium^{1/2} B5 (Gamborg et al. (1968) Exp. Cell Res. 50:151): 375 mg/l $CaCl_2 \cdot 2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodiumsuccinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzylaminopurin (BAP).

The protoplast suspension is mixed 1:1 with $\frac{1}{2}$ B5 culture medium with agarose. The agarose beads are plated in larger petridishes with liquid $\frac{1}{2}$ B5 culture medium on top of it.

- 25 The petri dishes are taped with parafilm and cultured at 25°C. After 6 days selection of the microcalli is performed by adding 500 mg/l of the selective agent spectinomycin dihydrochloride. One week after initiation of culture the culture medium is diluted with fresh liquid $\frac{1}{2}$ B5 culture medium, with addition of spectinomycin hydrochloride. When calli are about 0.5 mm in size they are transferred to callusgrowth medium SH2 (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 30 g/l saccharose, 5 g/l

agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations described above. After 2 weeks calli can be transferred to regeneration medium SHreg
5 (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 15 g/l saccharose, 15 g/l maltose, 0.1 mg/l NAA and 0.1 mg/l BAP and spectinomycin dihydrochloride in concentrations described above. Spectinomycin resistant calli will appear as green calli amongst white (non-resistant)
10 calli.

Regenerating plants will appear after approximately 6 weeks and furtheron, and can be transferred to rooting medium (Schenk and Hildebrandt, supra) with 30 g/l saccharose and 8 g/l agar with the concentrations of spectinomycin
15 dihydrochloride mentioned above. Alternatively, in transformation vectors where gfp (green fluorescent protein) is added as favourable gene apart from aadA, gfp fluorescence can be detected using an inverted microscope with the proper filter combinations.

20 Spectinomycin resistant calli were obtained after transformation of protoplasts with the plasmid pLCV2-MSK18. Approximately 40-50% of the protoplast will survive the PEG treatment. Three independent experiments yielded in total 7 resistant calli (see Table 1). These green calli were
25 detected 4-5 weeks after initiation of each experiment. Callus lines of each individual event are maintained on medium SHreg with the selective agent spectinomycin dihydrochloride.

For pLCV1-MSK18, no resistant calli were detected
30 as yet.

Spectinomycin resistance of plant cells may be the result, apart from transformation with the vector LCV2-MSK18, of spontaneous mutation of chloroplast DNA or insertion of

the DNA into the nuclear genome. Therefore, the callus was screened for the integration of the right and left homologous border segment (see example 9). Additionally, it was determined whether the *aadA* gene and the *gfp* gene was correctly integrated in the chloroplast DNA.

The transgenic callus has been obtained using vectors with specific lettuce chloroplast DNA homologous sequences. Selection of transformed cells with the non-lethal selective agent spectinomycin has been successful. The optimal transformation frequency for lettuce, determined as the number of green calli to the number of surviving protoplasts is about 1 in 6.10^6 protoplasts (Table 1, Exp 1 and 3).

Table 1. Selection of plastid transformants

Treatment	# pps treated	# green calli	# green calli showing regeneration
Exp 1			
Control	None	0	0
Control + PEG	1.26×10^6	0	0
pLCV1-MSK18	1.26×10^6	0	0
pLCV2-MSK18	1.26×10^6	1	0
Exp 2			
Control	None	0	0
Control + PEG	1.20×10^6	0	0
PLCV2-MSK18	2.40×10^6	1	0
Exp 3			
Control	None	0	0
Control + PEG	1.20×10^6	0	0
PLCV2-MSK18	4.80×10^6	5	2

EXAMPLE 6

Transformation of protoplasts via electroporation and selection on *aadA* encoded antibiotic resistance

Protoplasts, derived as described in example 3, are

suspended in transformation buffer HSB (150 mM KCL, 4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES (pH 7.2), and enough mannitol to osmotically balance the protoplasts. This is dependent on the genotype but it can easily be found out experimentally.

5 Aliquots of 1×10^6 protoplasts / 0.5 ml HSB buffer and mannitol are put into a conical centrifuge tube, and plasmid DNA solution is added. Plasmid DNA concentrations in the transformation buffer should preferably be in the range of 10-100 $\mu\text{g/ml}$. The protoplast-DNA suspension is transferred to
10 the electroporation chamber and electroporated using a single electric pulse (e.g. 325 μF , 300 V) The optimal setting can vary with species and cell type, and should be determined in preliminary experiments. The most efficient parameters are set by finding the pulse settings that result in 50%
15 protoplast death by 24h after the shocks. More details of the method are described by G.W. Bates ((1999), Plant transformation via protoplast electroporation. From: Methods in Molecular Biology Vol 111: Plant cell Culture Protocols, Pp 359-373).

20 After electroporation, protoplasts culture is performed as described in example 4.

EXAMPLE 7

Adjustment of spectinomycin threshold levels in cotyledons

25 For the adjustment of the optimal concentration of spectinomycin, for selection of cells with chloroplasts, which are transformed with constructs having the aadA gene as selectable marker, 4 day old cotyledons were plated on media with various concentrations of spectinomycin dihydrochloride.
30 The cotyledons were obtained as described in example 1.

EXAMPLE 8Transformation of plant material via biolistics and selection for aadA encoded antibiotic resistance

For bombardment of cotyledons, seeds were sown as described in example 1. Alternatively, leaf pieces can be used as explant material for shooting, under similar conditions. In this example, the use of cotyledons is described. Cotyledons (3 to 12 days old) are placed with the abaxial side onto MS1 medium (Murashige and Skoog, supra) with 30 g/l saccharose, 8 g/l agar and supplemented with 100-200 mg/l carbenicillin 0.1 mg/l benzylaminopurin (BAP) and 0.1 mg/l 1-naphthalene acetic acid (1-NAA) at pH 5.8. Gold particles (0.6 to 1.6 μ m) were prepared for transformation by mixing 50 μ l of suspension (60 mg/ml 50% glycerol) with 5 μ g DNA (1 μ g/ μ l H₂O), 50 μ l CaCl₂·2H₂O (2.5 M) and 20 μ l spermidine (0.1 M base). The particle-DNA mixture was incubated at room temperature for 1-3 minutes and centrifuged for 3-10 sec. in an Eppendorf centrifuge. After removal of the supernatant, the coated particles are washed and diluted in 48-60 μ l ethanol. The particles (6-8 μ l per carrier) are applied to the macrocarrier holders and the bombardment is performed with PDS-1000/He Biolistic particle delivery system (BioRad). Details of the procedure has been described by Klein et al. (Bio/Technology 6: 559-563 1988).

Two to fourteen days after bombardment, the cotyledons are transferred to MS1 medium as described above with the addition of a selective agent (e.g. spectinomycin dihydrochloride at concentration of 500 mg/l). Cultures are transferred onto fresh medium every 2 weeks. When green callus or shoots appear, they are transferred to medium MS2 without carbenicillin, but including the selective agent spectinomycin dihydrochloride.

EXAMPLE 9Molecular analysis of spectinomycin resistant calli of lettuce

Spectinomycin resistant callus of lettuce was
 5 analysed by PCR using different primer combinations to
 confirm the integration of the plasmid pLCV2-MSK18 in the
 genome of the chloroplast.

As an endogenous control for chloroplast DNA
 amplification, PCR analysis of the ATPase gene (Accession:
 10 AF162208) was carried out using the forward primer 5'-
 ACTAATAGTGGACAAATTGGC-3' and the reverse primer 5'-
 TTGCTTGATTGTATTTACTCG-3'. To detect the presence of the
 selectable marker gene Aada, the following primer combination
 was used: forward 5'-TATGACGGGCTGATACTGGGC-3' and reverse 5'-
 15 AAGTCACCATTTGTTGTGCACG-3'. In order to demonstrate the
 physical integration of the plasmid into the chloroplast
 genome 2 primer combinations were developed which amplify
 hybrid regions of the plasmid and the chloroplast genome. The
 first primer combination consisting of forward 5'-
 20 ACTGGAAGGTGCGGCTGGAT-3' and reverse 5'-
 ACGAGCCGGATGATTAATTGTCAATTAATTAATA-3' amplifies the junction
 containing the trnI sequence of the chloroplast genome. The
 second primer combination consisting of forward 5'-
 AAGTCACCATTTGTTGTGCACG -3' and reverse 5'-
 25 CTCGCCCTTAATTTTAAGGC-3' amplifies the junction containing the
 trnA sequence of the chloroplast genome.

Total DNA was isolated from spectinomycin resistant callus
 using a commercially available DNA isolation kit from Sigma
 (Genelute Plant Genome DNA Kit). The PCR reaction was carried
 30 out using a total amount of 30 ng DNA after which the
 reaction products were analysed on a 1% agarose gel. The
 result of the analysis of 5 independent spectinomycin
 resistant calli is shown in Figure 1. The ATPase fragment of

about 424 bp is only present in callus material and leaf material of lettuce, and as expected not visible for the pLCV MSK18 DNA. PCR amplification of the *aadA* gene gave the expected fragment of approximately 413 bp for the transgenic callus and the plasmid pLCV2-MSK18. To confirm the integration of the pLCV2-MSK18 vector into the lettuce chloroplast genome, the two primer combinations were used which specifically detect either one of the two junctions which emerge after integration of the plasmid by homologous recombination. Figure 1 D shows the amplification of the *trnA* junction which results in an expected band of approx. 1500 bp in the spectinomycin resistant callus. The integration on *trnI* junction was investigated using the PC indicated above which resulted in an expected band of approximately 1500 bp as well. The results of this analysis confirm the transplastomic nature of the obtained spectinomycin resistant lettuce calli.

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CLAIMS

1. Method for the transfromation of plant plastids
as described in the description.

5 2. Compositions for use in the method as claimed in
claim 1.

Figure 1. LCV1 lettuce chloroplast genome target sequence (not including backbone vector).

GTTCAAGAATCAGTTTTCTTTTTATAAGGGCTAAAATCACTTATTTTGGCTTTTTTACCCCATATTGTAGGGTG
GATCTCGAAAGATATGAAAGATCTCCCTCCAAGCCGTACATACGACTTTTCATCGAATACGGCTTTCCGCAGAAT
TCTATATGTATCTATGAGATCGAGTATGGAATCTGTTTACTCACTTTAAATTTAGATATCCGTTTCCCTCCTTT
TCCTGCTAGGATTGGAAATCCTGTATTTTACATATCCATACGATTGAGTCCTTGGGTTTCCGAAATAGTGTA
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ACACGGACAAAGTCAGGGAACCTCTGAAATTTTTTCAATATTGAACCTTGGACATATAATAGTTCCGAATCG
AATCTCTTTAGAAAGAAGATCTTTGTCTCATGGTAGCCTGCTCCAGTCCCCTTACGAACTTTTCGTTATTGGG
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ATTTTCACTCATACGGCTCCTCGTTCAATTCTTTCGAAGTTATTGGATCCTTTTCCGCGTTCGAGAATCCCC
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ATTTAAAGCACTATGTGAAATATTCGGTTTTTCTCTCTATCCCATAGGTACAGTGTGTAATCAATCG
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AATTGGATCCTAAATGACGGGTTAGTGTGAGCTTATCCATGCGGTTATGCACTCTTCAATAGGAATCCATTT
TCTGAAAGATCCTGGCTTTCGTGCTTTGGTGGGCTCTCCGAGATCCTTTTCGATGACCTATGTTGTGTTTGTGAA
GGGATATCTATATAATACGATCGATTGCGTAAAGCCCGCGGTAGCAGTGGAACCGGGGAAAGTATACAGAAAAG
ACAGTTCTTTTCTATTATATATTATATTAGTCTTTTCTATTATTCATATTAGATTAGTCTTAGTTAGTGATC
CCGGCTTAGTGAGTCTTCTTCCGTGATGAAGTGTGGCGCCAGTCTTACATTTTGTCTCTGTGGACAGAGGA
GAAAGGGGCTCCGCGGGAAGAGGATTGTACCGTGAGAGAAGCAAGGAGGTCAACCTCTTCAAATATACAACA
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TTTGCCTGTTAGGTAACAGGATAGCAAGTTACAACTCTGTCTCGGTAGGACATGGATCTCTATTACTATGAAT
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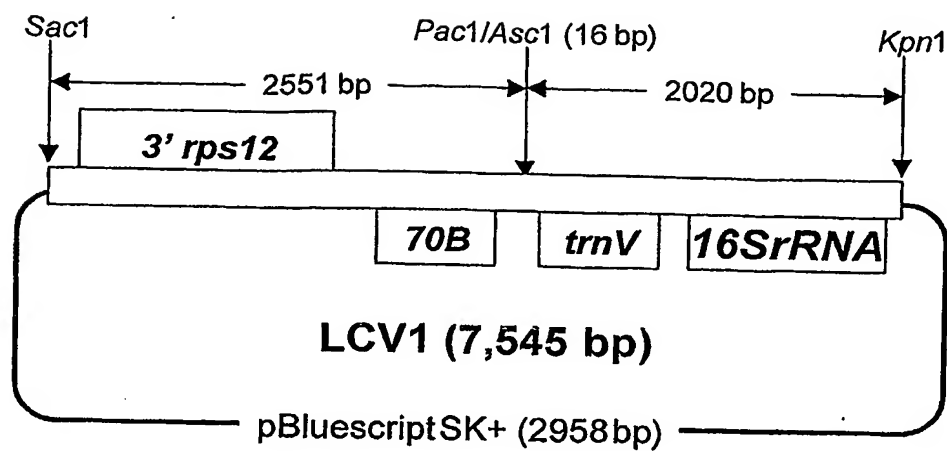


Figure 2. Map of LCV1 (7,535 bp).

Figure 3. LCV1 lettuce chloroplast genome target sequence aligned with tobacco chloroplast genome (GI Z00044).

LCV1:	1	gttcaagaatcagttttctttttataagggctaaaatcacttattttggcttttttacc	60
tobac:	100021	gttcaagaatcagttttctttttataagggctaaaatcacttattttggcttttttacc	100080
ribosomal protein S12	80	^^^ K P K K V G	
LCV1:	61	catattgtagggtggatctcgaaagatatgaagatctccctccaagccgtacatacgac	120
tobac:	100081	catattgtagggtggatctcgaaagatatgaagatctccctccaagccgtacatacgac	100140
ribosomal protein S12	78	Y K ~~~~~	
LCV1:	121	tttcatcgaatacggctttccgcagaattctatatgtatctatgagatcgagtatggaat	180
tobac:	100141	tttcatcgaatacggctttccgcagaattctatatgtatctatgagatcgagtatggaat	100200
ribosomal protein S12	1	~~~~~	
LCV1:	181	tctgtttactcacttttaaattgagtatccgtttccctcccttttctgctaggattggaaa	240
tobac:	100201	tctgtttactcacttttaaattgagtatccgtttccctcccttttctgctaggattggaaa	100260
ribosomal protein S12	1	~~~~~	
LCV1:	241	tcctgtattttacatatccatacgattgagtccttgggtttccgaaatagtgtaaaaaga	300
tobac:	100261	tcctgtattttacatatccatacgattgagtccttgggtttccgaaatagtgtaaaaaga	100320
ribosomal protein S12	1	~~~~~	
LCV1:	301	agtgcctcaaatacattgctatttgactcggacctgttctaaaaa-gtcgaggtatttcga	359
tobac:	100321	agtgcctcgaatacattgctatttgactcggacctgttctaaaaaagtcgaggtatttcga	100380
ribosomal protein S12	1	~~~~~	
LCV1:	360	attgtttgttgacacggacaaagtcagggaaaacctctgaaatttttcaatattgaacc	419
tobac:	100381	attgtttgttgacacggacaaagtcagggaaaacctctgaaatttttcaatattgaacc	100440
ribosomal protein S12	1	~~~~~	
LCV1:	420	ttggacatataatagttccgaatcgaatctctttagaagaagatctttgtctcatggt	479
tobac:	100441	ttggacatataatagttccgaatcgaatctctttagaagaagatctttgtctcatggt	100500
ribosomal protein S12	1	~~~~~	
LCV1:	480	agcctgctccagtcaccttacgaaactttcggtattgggttagccatacacttcacatgt	539
tobac:	100501	agcctgctccagtcaccttacgaaactttcggtattgggttagccatacacttcacatgt	100560
ribosomal protein S12	1	~~~~~	
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tobac:	100561	ttctagcgattcacatggcatcatcaaatgatacaagtcttggataagaatctacaacgc	100620
ribosomal protein S12	1	~~~~~	
LCV1:	600	actagaacgccttgttgacgatcctttactccgacagcatctagggttcctcgacaat	659
tobac:	100621	actagaacgccttgttgacgatcctttactccgacagcatctagggttcctcgacaat	100680
ribosomal protein S12	59	~~ S R G Q Q R D K V G V A D L T G R V I	
LCV1:	660	gtgatatctcacaccgggtaaatccttaaccctccccctcttactaagactacagaatg	719

tobac: 100681 gtgatatctcacaccgggtaaatccttaacccttccccctcttactaagactacagaatg 100740
ribosomal protein S12 39 H Y R V G P L D K V R G G R V L V V S H

LCV1: 720 ttcttgtaattatggccaataaccgggtatataagcagtgatttcaaataccagaggtaa 779
tobac: 100741 ttcttgtaattatggccaataaccgggtatataagcagtgatttcaaataccagaggtaa 100800
ribosomal protein S12 19 E Q L N H G I G P I Y A T I E F G S T L

LCV1: 780 tcgtactctggcaactttacgtaaggcagagtttggttttttgggggtgatagtgaaaa 839
tobac: 100801 tcgtactctggcaactttacgtaaggcagagtttggttttttgggggtgatagtgaaaa 100860
ribosomal protein S12 1 R V R A V K R L A S N P K K P T I T

LCV1: 840 gttgacagataagtcacccttactgccactctacagaaccgtacatgagattttcacctc 899
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LCV1: 900 atacggctcctcggtcaattctttcgaagttattggatccttttccgcgttcgagaatcc 959
tobac: 100921 atacggctcctcggtcaattctttcgaagttattggatccttttccgcgttcgagaatcc 100979

LCV1: 960 cctcccttcttccactccgtcccgaagagtaactaggaccaatttagtcacgttttcatg 1019
tobac: 100980 cc-ccttcttccactccgtcccgaagagtaactaggaccaatttagtcacgttttcatg 101038

LCV1: 1020 ttccaattgaacactttccggtttt-----
tobac: 101039 ttccaattgaacactgtccatttttgattattctcaaaggataa 101082

LCV1: 1045 gattattctctttaccaaacatatgcggatccaatcacgatcttata----ataagaaca 1100
tobac: 101083 gattattctctttaccaaacatatgcggatccaatcacgatcttataataagaagaaca 101142

LCV1: 1101 agagatctttctcgatcaatcccttgccctcattcttctcgagaatcagaaagatccttt 1160
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LCV1: 1161 tcaagtttgaatttggtcatttggatctgagttcttctacttcattattttatttaatat 1220
tobac: 101203 tcaagtttgaatttggtcatttggatctgagttcttctacttcattattttatttaatat 101261

LCV1: 1221 caatattttgcctctcttttttttatatttcccttaagtcccataggtttgatccttt 1280
tobac: 101262 gaatattttc-cctctcttttttttatatttcccttaagtcccataggtttgatcctgt 101320

LCV1: 1281 agaattggactcattttctcattgagcgaagggtacgaaataaatcagattgattaaaag 1340
tobac: 101321 agaatttgaccattttctcattgaacgaaagggtacgaaataaatcagattgat-aaaag 101379

LCV1: 1341 cactatgtgaaatattcggttttt-----tcctcttctctatcccataggt-----aca 1390
tobac: 101380 taccatgtgaaatcttcggtttttcccttctctcgatccctatcccataggttaggtaca 101439

LCV1: 1391 gtgtttgaatcaatcgagaaccttttctctgtctgaatcgatattattccattccaatt 1450
tobac: 101440 gtgtttgaatcaatcgagaaccttttctctgtctgaatcgatattattccattccaatt 101499

LCV1: 1451 ccttccccgatacctctcaaggaaaatctcgaatt-ggatacctaaattgacgggttagtgt 1509
 |||||
 tobac: 101500 ccttccccgatacctcccaaggaaaatctcgaatttgatcccaattgacgggttagtgt 101559
 |||||

LCV1: 1510 gagcttatccatgcggttatgcactcttcgaataggaatccattttctgaaagatcctgg 1569
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LCV1: 1570 ctttcgtgctttggtgggtctccgagatcctttcgatgacctatgttgtgtttgttgaag 1629
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LCV1: 1630 ggatatctatataatacgcgattgcgtaagcccgcggttagcagtgggaaccggggaaa 1689
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LCV1: 1690 gtatacagaaaagacagtcttttctatttat 1722
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LCV1: 1723 attatattagctcttttctatttaattc 1749
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LCV1: 1810 tgttggcgccagtcctacattttgtctctgtggacagaggagaaaaaggggtccgcggga 1869
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LCV1: 1870 agaggattgtaccgtgagagaagcaaggagggtcaacctctttcaaatatacaacatggat 1929
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 hypothetical protein 127 ^^^ I Y L M S

LCV1: 1930 tctggcaatgcaatgtacttggactctcatgtcgatccgaatgaatcatcctttccacgg 1989
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 tobac: 101970 tctggcaatg-----tagttggactctcatgtcgatccgaatgaatcatcctttccacgg 102024
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LCV1: 1990 aggcaaatctttgcctgttaggtaacaggatagcaagttacaaactctgtctcggttagga 2049
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 tobac: 102025 aggtaaatctttgcctgttaggtaacaggatagcaagttccaaattctgtctcggttagga 102084
 hypothetical protein 88 T F R Q R S P L L I A L E L N Q R P L V

LCV1: 2050 catggatctctattactatgaatttcataaatgaagtagtgaatgggtgggttaccatta 2109
 |||||
 tobac: 102085 catgtatttctattactatgaatttcataaatgaagtagtgaatgggtgggttaccatta 102144
 hypothetical protein 1 M K F I N E V V N G R V T I
 hypothetical protein 68 H I E I V I F N M F S T T L P L T V M I

LCV1: 2110 tcctttttgtagtgacgaatcctgtatgtgttcctaagaaaaggaatttgcatttttc 2169
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 tobac: 102145 tcctttttgtagtgacgaatcctgtatgtgttcctaagaaaaggaatttgcatttttc 102204
 hypothetical protein 15 I L F V V T N L V C V P K K R N L S I F
 hypothetical protein 48 R K T T V F R T H T G L F L F K D M K R

|||
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 LCV1: 3108 acggctgctaataaccccgtaggctgagagcaaaaggaggaatccgcccaggaggggct 3167
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LCV1: 3888 aaccttaccagggccttgacatgccgcgaatcctcttgaagagaggggtgccttcgggaa 3947
 |||
 tobac: 103685 aaccttaccagggccttgacatgccgcgaatcctcttgaagagaggggtgccttcgggaa 103744

LCV1: 3948 cgcggacacaggtggtgcatggctgtcgtcagctcgtgccgtaagggtgttgggttaagtc 4007
 |||
 tobac: 103745 cgcggacacaggtggtgcatggctgtcgtcagctcgtgccgtaagggtgttgggttaagtc 103804

LCV1: 4008 ccgcaacgagcgcaaccctcgtgtttagttgccatcattgagtttgaaccctgaacaga 4067
 |||
 tobac: 103805 ccgcaacgagcgcaaccctcgtgtttagttgccatcgttgagtttgaaccctgaacaga 103864

LCV1: 4068 ctgccggtgataagccggaggaaggtaggatgacgtcaagtcacatgcccccttatgcc 4127
 |||
 tobac: 103865 ctgccggtgataagccggaggaaggtaggatgacgtcaagtcacatgcccccttatgcc 103924

LCV1: 4128 ctgggcgacacacgtgctacaatggccgggacaaagggtcgcgatcccgcgaggggtgagc 4187
 |||
 tobac: 103925 ctgggcgacacacgtgctacaatggccgggacaaagggtcgcgatcccgcgaggggtgagc 103984

LCV1: 4188 taacccccaaaaaccgctcctcagttcggtatgcaggctgcaactcgctcatgaagccg 4247
 |||
 tobac: 103985 taacccccaaaaaccgctcctcagttcggtatgcaggctgcaactcgctcatgaagccg 104044

LCV1: 4248 gaatcgctagtaatcgccggtcagccatagcgcggtgaatccgttcccgggccttgtagc 4307
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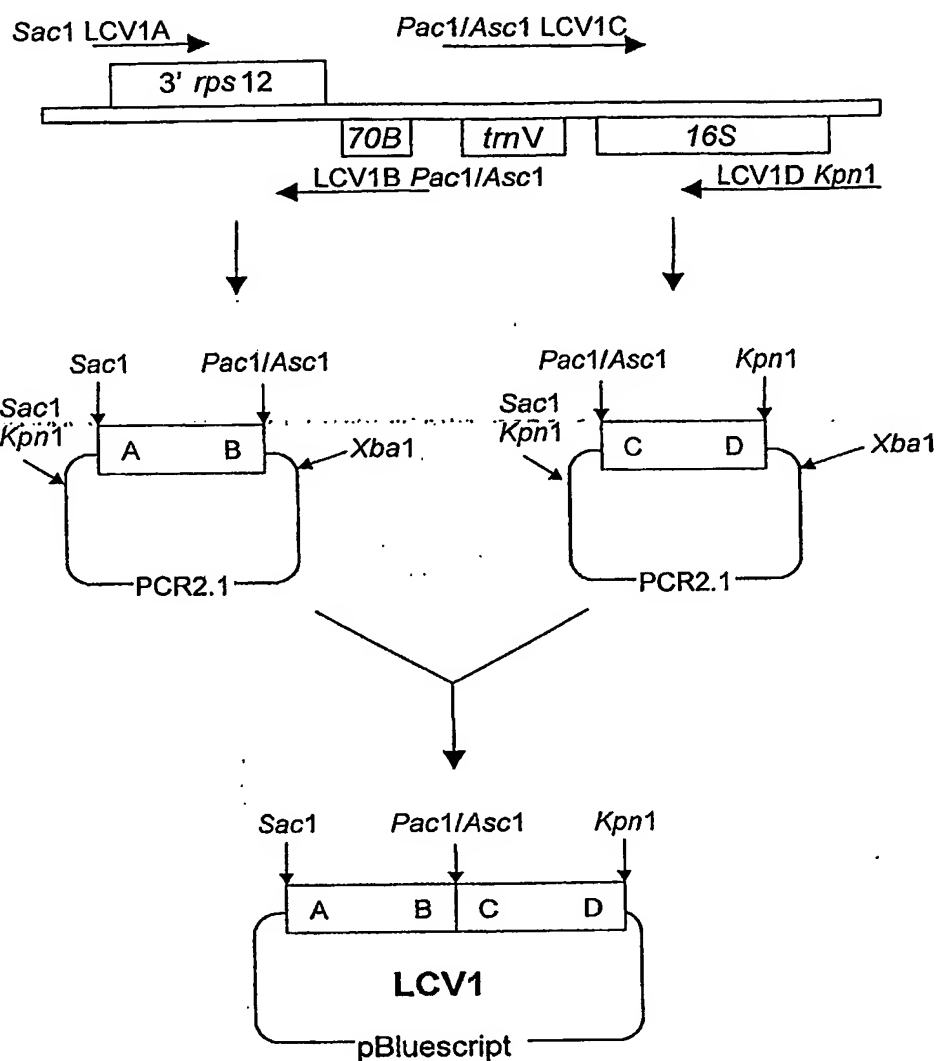
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 |||
 tobac: 104105 caccgcccgtcacactatgggagctggccatgcccgaaagtcgttaccttaaccgcaagga 104164

LCV1: 4368 gggggatgccgaaggcagggctagtactggagtgaagtcgtaacaaggtagccgtactg 4427
 |||
 tobac: 104165 gggggatgccgaaggcagggctagtactggagtgaagtcgtaacaaggtagccgtactg 104224

LCV1: 4428 gaaggtgctggctggatcacctccttttcaggagagctaagtcttgggtattttggt 4487
 |||
 tobac: 104225 gaaggtgctggctggatcacctccttttcaggagagctaagtcttgggtattttggt 104284

LCV1: 4488 ttgacactgcttcacaccc-----aaaaaagaaggagctacgtctgagttaaacttgag 4543
 |||
 tobac: 104285 ttgacactgcttcacacccccaaaaaagaaggagctacgtctgagttaaacttgag 104344

LCV1: 4544 atggaagtcttcatttcgtttctcgacagtgaagtaagaccaag 4587
 |||
 tobac: 104345 atggaagtcttc-tttcctttctcgacggtgaagtaagaccaag 104387



LCV1A-5' ATGAGCTCGTTCAAGAATCAGTTTTCTT3' (100021-100040 in TCG)
 LCV1B-5' GGCGCGCCTTAATTAATCTTTTTTTTCGCACTATTACGGATAT3' (102345-102367 in TCG)
 LCV1C-5' TTAATTAAGGCGCGCCAGGCCCGGCCCAAGTT3' (102368-102384 in TCG)
 LCV1D-5' ATGGTACCCTTGGTCTTACTTCACTGTCTGA3' (104366-104387 in TCG)

Figure 4. Cloning steps and primers for construction of LCV1. TCG = tobacco chloroplast genome.

Figure 5. LCV2 lettuce chloroplast genome target sequence (not including backbone vector).

TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTTTT
ACGTCCCCATGCCCCCTGTGTGGCGACATGGGGGCGAAAAAGGAAAGAGAGATGGGGTTTCTCTCGCTTTT
GGCATAGTGGGCCCCCAGTGGGGGGCTCGCACGACGGGCTATTAGCTCAGTGGGTAGAGCGCGCCCTGATAAT
TGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATCGGCGCCTGACCCT
GAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTGTTTCAACCGGGGTTTGAAACCAAACCTTC
TCCTCAGGAGGATAGATGGGGCGATTGAGGTGAGATCCAATGTAGATCCAACCTTCGATTCACTCGTGGGATCC
GGCGGTCCGGGGGGGACCAACCATGGCTCCTCTCTCTCGAGAATCCATACATCCCTTATCAGTGTATGGACAG
CTATCTCTCGAGCACAGGTTTAGGTTTCGGCCTCAATGGGAAAATAAAATGGAGCACCTAACACGCATCTTCAC
AGACCAAGAACTACGAGATCACCCCTTTTCATTCTGGGGTGACGGAGGGATCATACCATTTCGAGCCTTTTTTTT
CATGCTTTTCCCCGAGGTCTGGAGAAAGCTGAAATCAATAGGATTTCCCTAATCCTCCCTTACCGAAAGGAAGA
GCGTGAAATCTTTTTTCTTTCCGCGAGGGACCAGGAGATTGGATCTAGCCGTAAGAAGAATGCTTGGTATAAAT
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CTCTTAACGCCTTCTTTTTTCTTCTCATCGGAGTTATTTACAAAGACTTGCCATGGTAAGGAAGAAGGGGGGA
ACAGGCACACTTGGAGAGCGCAGTACAACGGAGAGTTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAA
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TCAAGTCCAGGATGGCCAGCTGCGCCAGGGAAAAGAATAGAAGAAGCGTCAGACTATTAATTAAAGGCGCGCCC
ATGCATGCTCCACTTGGCTCGGGGGGATATAGCTCAGTTGGTAGAGCTCCGCTCTTGCAATTGGGTCGTTGCGA
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GCACTATCTCCCTTCAACCCCTTTGAGCGAAATGCGGCAAAAGGAAGGAAAATCCATGGACCGACCCCATCATCT
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CTGTTCAATAAGTGGAACGCATTAGCTGTCCGCTCTCAGGTTGGGCAGTAAGGGTCGGAGAAGGGCAATCACTC
ATTCTTAAAACAGCGTTCTTAAGGCCAAAGAGTCGGCGGAAAAGGGGGGAAAGCTCTCCGTTCTTGGTTTCCT
GTAGCTGGATCCTCCGGAACCACAAGAATCCTTAGTTAGAATGGGATTCCAACCTCAGCACCTTTTGAGTGAGAT
TTTGAGAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTGGGGGGGAGTTATTGTC
TATCGTTGGCCTCTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGTGGTTTACCCTGCGGCGGATGTCAGCG
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TTGGCGGTTCGATCTATGATTTATCATTCATG

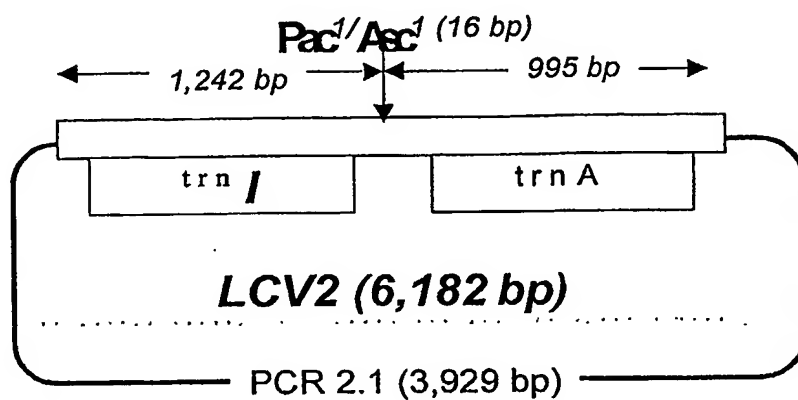


Figure 6. Map of LCV2 (6,182 bp).

LCV2 : 1 tcgacagtggaagtaagaccaagctcatgagcttattatctcaggtcgggaacaagttgata 60
|||||
tobac: 104366 tcgacggtgaagtaagaccaagctcatgagcttattatcctaggtcgggaacaagttgata 104425
|||||

LCV2 : 61 ggatccccctttttacgtcccatg--ccccctgtgtggcgacatggggcgaaaaaagg 118
||| |||
tobac: 104426 ggacccccctttttacgtcccatgttccccctgtgtggcgacatggggcgaaaaaagg 104485
|||||

LCV2 : 119 aaagagagagatgggggtttctctcgcttttggcatagtgggccccccagtggggggctcgc 178
|||||
tobac: 104486 aaagagagggatgggggtttctctcgcttttggcatagcgggccccccagtgggaggctcgc 104545
|||||

LCV2 : 179 acgacgggctattagctcagtgggtagagcgccccctgataattgcgtcgttgtgcctg 238
|||||
tobac: 104546 acgacgggctattagctcagtggttagagcgccccctgataattgcgtcgttgtgcctg 104604
|||||

LCV2 : 239 ggctgtgagggctctcagccacatggatagttcaatgtgctcatcgggcgctgacctga 298
|||||
tobac: 104605 ggctgtgagggctctcagccacatggatagttcaatgtgctcatcgggcgctgacctga 104664
|||||

LCV2 : 299 gatgtggatcatccaaggcacattagcatggcggtactcctcctgttcgaaccgggggttg 358
|||||
tobac: 104665 gatgtggatcatccaaggcacattagcatggcggtactcctcctgttcgaaccgggggttg 104724
|||||

LCV2 : 359 aaaccaaacttctcctcaggaggatagatggggcgattcaggtgagatccaatgtagatc 418
|||||
tobac: 104725 aaaccaaactcctcctcaggaggatagatggggcgattcgggtgagatccaatgtagatc 104784
|||||

LCV2 : 419 caactttcgattcactcgtgggatccgggcggtccgggggggaccaccatggctcctctc 478
|||||
tobac: 104785 caactttcgattcactcgtgggatccgggcggtccgggggggaccaccaggtcctcctc 104844
|||||

LCV2 : 479 ttctcgagaatccatacatcccttatcagtgtagtgagagctatctctcgagcacaggtt 538
|||||
tobac: 104845 ttctcgagaatccatacatcccttatcagtgtagtgagagctatctctcgagcacaggtt 104904
|||||

LCV2 : 539 taggttcggcctcaatgggaaaaataaaatggagcacctaacaacgcattcttcacagacca 598
|||
tobac: 104905 tag-----caatgggaaaaataaaatggagcacctaacaacgcattcttcacagacca 104955
|||

LCV2 : 599 agaactacgagatcacccctttcattctggggtgacggagggatcataccattcgagcc 657
|||||
tobac: 104956 agaactacgagatcgccctttcattctggggtgacggagggatcgtagcattcgagcc 105014
|||||

LCV2 : 658 ttttttttcatgcttttccccgaggtctggagaaagctgaaatcaataggatttccta 717
|||||
tobac: 105015 gtttttt----- 105021
|||||

atcctcccttaccgaaaggaagagcgtgaaattcttttcccttccgcaggaccaggagattggatctagccgtaagaagaatgcttg
gtataaataactcacttcttggtcttcgacccccgcagtcactacgaacgccccgatcagtgcaatgggatgtgtctatttatctatc
95
231 bp present in lettuce maize, rice and soybean but not tobacco)

LCV2 : 896 tcttgactcgaaatgggagcaggtttgaaaaggatcttagagtgtctagggttgggcca 955
 |||||
 tobac: 105022 tcttgactcgaaatgggagcaggtttgaaaaggatcttagagtgtctagggttgggcca 105081

LCV2 : 956 ggaggggtctcttaacgccttctttttctctcatcgagttatttcacaaagacttgcc 1015
 |||||
 tobac: 105082 ggaggggtctcttaacgccttctttttctctcatcgagttatttcacaaagacttgcc 105141

LCV2 : 1016 atggtaaggaagaaggggggaacaggcacacttgagagcgagtcacaacggagagttgt 1075
 |||||
 tobac: 105142 agggtaaggaagaaggggggaacaggcacacttgagagcgagtcacaacggagagttgt 105201

LCV2 : 1076 atgctgcgttcgggaaggatgaatcgctcccgaagaatctattgattctctccaat 1135
 |||||
 tobac: 105202 atgctgcgttcgggaaggatgaatcgctcccgaagaatctattgattctctccaat 105261

LCV2 : 1136 tgggtggaccgtaggtgcatgatttacttcacgggaggtctctggttcaagtccagg 1195
 |||||
 tobac: 105262 tgggtggaccgtaggtgcatgatttacttcacgggaggtctctggttcaagtccagg 105321

LCV2 : 1196 atggcccagctgcgccagggaagaatagaagaagcgctcagactccttaattaaggcgccgc 1258
 |||||
 tobac: 105322 atggcccagctgcgccagggaagaatagaagaagcatctgactactt-----105370

LCV2 : 1259 catgcatgctccacttggtcggggggatatagctcagttggtagagctccgctcttgca 1318
 |||||
 tobac: 105371 catgcatgctccacttggtcggggggatatagctcagttggtagagctccgctcttgca 105430

LCV2 : 1319 attgggtcggttgcatgattacgggttggtatgtctaattgtccaggcggtaatgatagtatct 1378
 |||||
 tobac: 105431 attgggtcggttgcatgattacgggttggtatgtctaattgtccaggcggtaatgatagtatct 105490

LCV2 : 1379 tgtacctgaaccggtgggtcactttttctaaagtaatggggaagaggaccgaaacatgcc 1438
 |||||
 tobac: 105491 tgtacctgaaccggtgggtcactttttctaaagtaatggggaagaggaccgaaacatgcc 105550

LCV2 : 1439 ctgaaagactctactgagacaaagatgggctgtcaagaacgtcaagaacgtagaggaggt 1498
 |||||
 tobac: 105551 ctgaaagactctactgagacaaagatgggctgtcaagaa-----cgtagaggaggt 105601

LCV2 : 1499 aggatgggcagttgggtcagatctagtatggatcggtacatggacggtagttggagtcggcg 1558
 |||||
 tobac: 105602 aggatgggcagttgggtcagatctagtatggatcggtacatggacggtagttggagtcggcg 105661

LCV2 : 1559 gctctcctagggttcccttatcggggatccctggggaagaggatcaagttggcccttgog 1618
 |||||
 tobac: 105662 gctctccagggttccctcatctgagatctctggggaagaggatcaagttggcccttgog 105721

LCV2 : 1619 aacagcttgatgcactatctcccttcaaccctttgagcgaaatgcggc-----aaaagga 1673
 |||||
 tobac: 105722 aacagcttgatgcactatctcccttcaaccctttgagcgaaatgcggcaaaagaaaagga 105781

LCV2 : 1674 aggaaaatccatggaccgaccccatcatctccaccccgtaggaactacgagattacccca 1733
 |||||
 tobac: 105782 aggaaaatccatggaccgaccccatcatctccaccccgtaggaactacgagattacccca 105841

LCV2 : 1734 aggacgccttcggcatccaggggtcacggaccgaccatagaaccctgttcaataagtgg 1793

|||||
tobac: 105842 aggacgccttcggcatccaggggtcacggaccgaccatagaaccctgttcaataagtga 105901

LCV2 : 1794 acgcattagctgtccgctctcaggttgggcagtaagggtcggagaagggaatcactcat 1853
|||||

tobac: 105902 acgcattagctgtccgctctcaggttgggcagtcagggtcggagaagggaatgactcat 105961

LCV2 : 1854 tctta 1858
|

tobac: 105962 t---- 105962

LCV21859aaaccagcgttcttaaggccaaagagtcggcggaaaagggggaaagctctccgttcctggtttcctgtagctggatcctc
cggaaccacaagaatc 1955 (97 bp sequence absent in tobacco but present in spinach, Solanum
nigrum, Arabidopsis, Soybean, rice and wheat)

LCV2 : 1956 cttagttagaatgggattccaactcagcaccttttgagtgagattttgagaagagttgct 2015
|||||

tobac: 105963 cttagttagaatgggattccaactcagcaccttttgagtgagattttgagaagagttgct 106022

LCV2 : 2016 ctttgagagcacagtacgatgaaagttgtaagctgtgttcgggggggagttattgtcta 2075
|||||

tobac: 106023 ctttgagagcacagtacgatgaaagttgtaagctgtgttcgggggggagttattgtcta 106082

LCV2 : 2076 tcggtggcctctatggttagaatcagtcgggggacctgagaggcggtggtttaccctgcgg 2135
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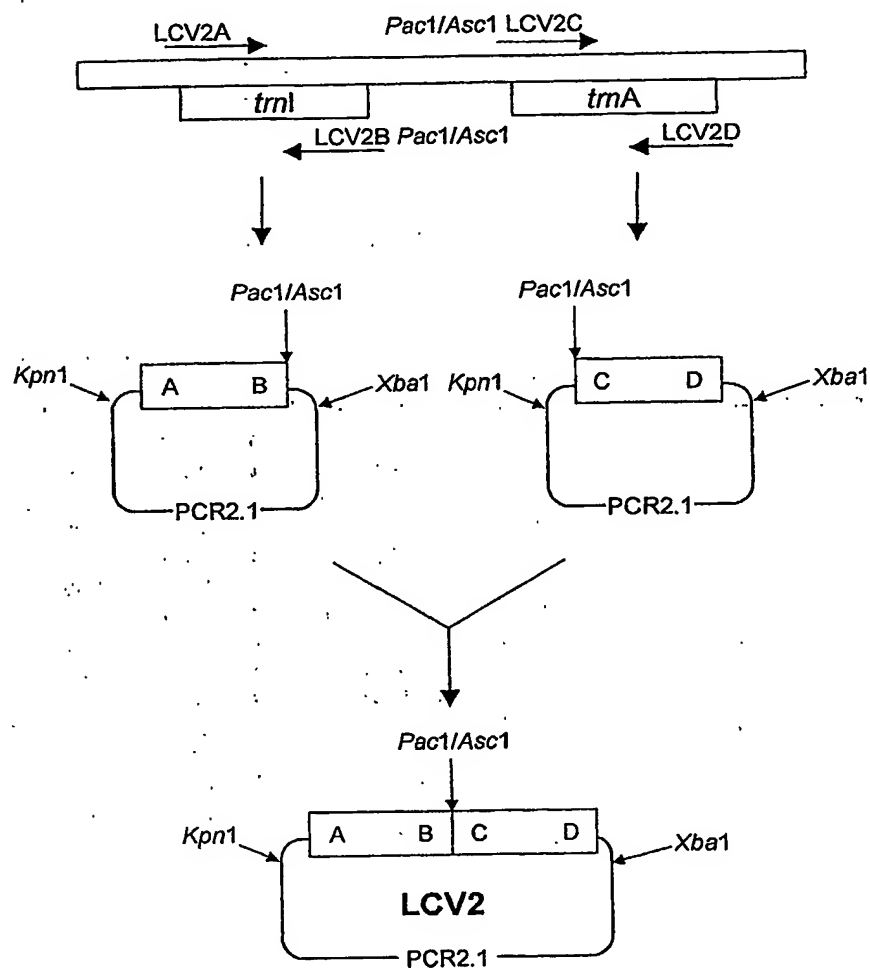
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LCV2 : 2136 cggatgtcagcgggttcgagtcggttatctccaactcgtgaacttagccgatacaaaagct 2195
|||||

tobac: 106143 cggatgtcagcgggttcgagtcggttatctccaactcgtgaacttagccgatacaaaagct 106202

LCV2 : 2196 atatgacagcacccaatttttccgatttggcggttcgatctatgatttatcattcatg 2253
|||||

tobac: 106203 ttatgatagcacccaatttttccgattcggcggttcgatctatgatttatcattcatg 106260



LCV2A 5' TCGACAGTGAAGTAAGACCAAG3' (104366-104387 in TCG)
 LCV2B 5' GGCGCGCCTTAATTAAGGAGTCAGACGCTTCTTCTATTC3' (10346-105370 in TCG)
 LCV2C 5' TTAATTAAGCGCGCCCATGCATGCTCCACTTGGCTCGG3' (105371-105393 in TCG)
 LCV2D 5' CATGAATGATAAATCATAGATCGAAC3' (106234-106260 in TCG)

Figure 8. Cloning steps and primers for construction of LCV2. TCG= tobacco chloroplast genome.

LCV1-MSK18 map (9,682bp)

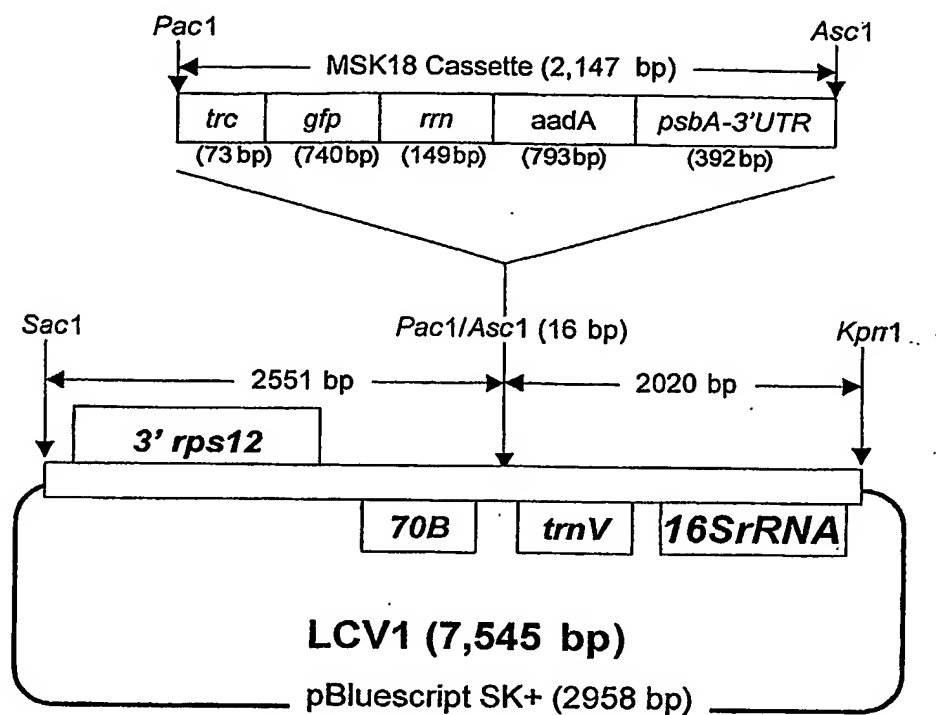


Figure 9. Map of LCV1 MSK18 (9,692 bp).

LCV2-MSK18 (8,329bp)

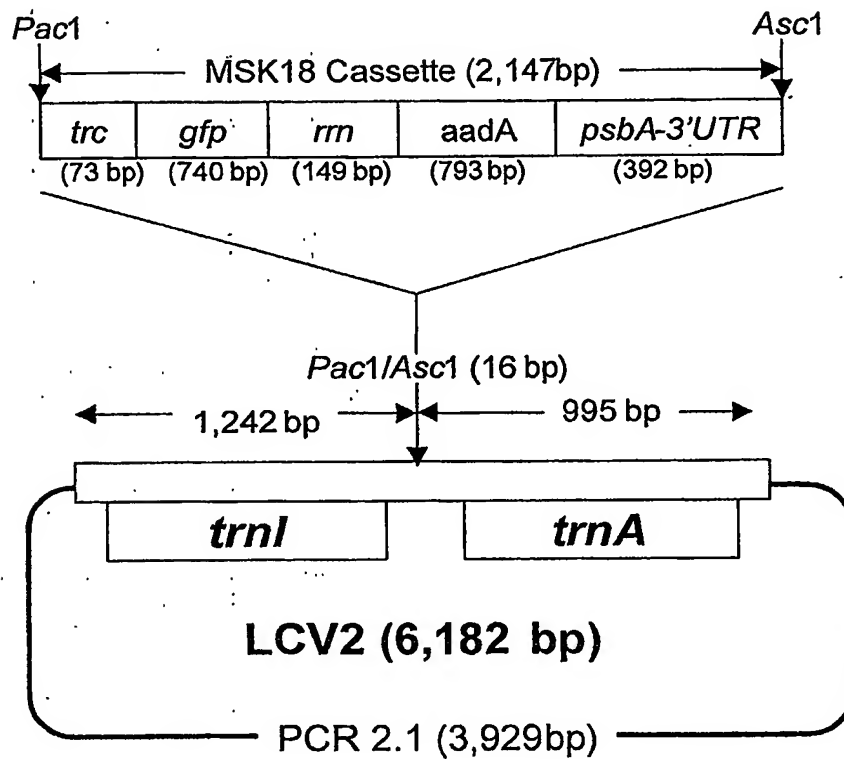
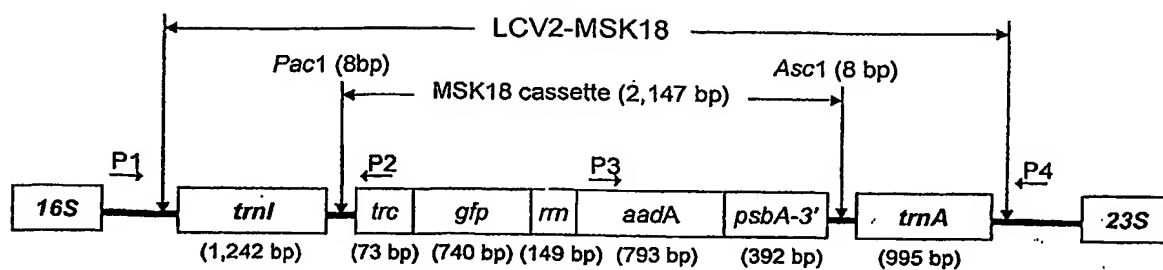


Figure 10. Map of LCV2-MSK18 (8,329 bp).



P1 + P2 = 1415 bp

P3 + P4 = 2006 bp

P1 + P4 = 4623 bp

P1 5'-ACTGGAAGGTGCGGCTGGAT-3'

P2 5'-ACGAGCCGGATGATTAATTGTCAATTAATTAATACTA-3' (MSK18A comp)-

P3 5'-AAGTCACCATTGTTGTGCACG-3' (starts at 259 bp on aadA CDS)

P4 5'-CTCGCCCTTAATTTTAAGGC-3'

Figure 11. Primer combinations used in PCR analysis of transplastomic lettuce callus.

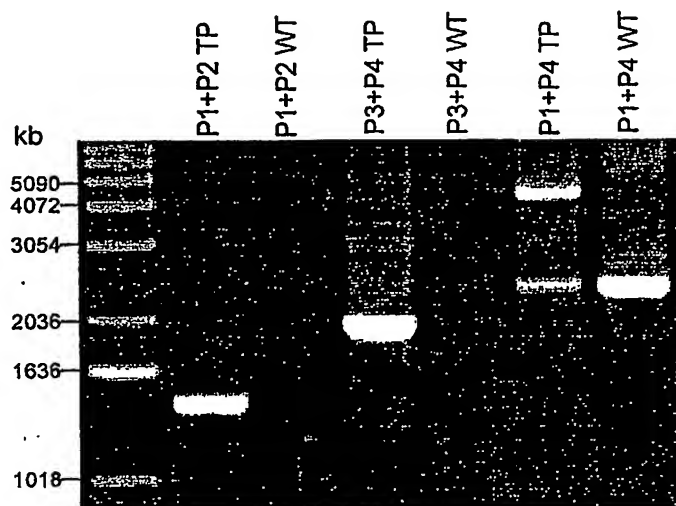


Figure 12. Agarose gel electrophoresis of PCR products from reactions with primer pairs P1+P2, P3+P4 and P1+P4 and template DNA from spectinomycin resistant putative transplastomic callus sample B (TP) and non-transformed wild-type callus (WT).

Figure 13. Sequence of left border (P1-P2) and right border (P3-P6) integration junction fragments amplified by PCR from transplastomic lettuce DNA. Sequence in lower case is lettuce chloroplast DNA external to the LCV2 vector target region.

P1-P2 left border fragment consensus sequence

Primer P1→

actggaaggtgcggtggatcacctccttttcagggagagctaattgcttggtgggtattttggtttgacac
tgcttcacaccccaaaaaagaagggagctacgtctgagttaaacttgagatggaagtcttcatttcgtttc

Primer LCV2A→=LCV2A left border

TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTT
TTTACGTCCCCATGCCCTGTGTGGCGACATGGGGGCGAAAAAGGAAAGAGAGAGATGGGGTTTCTCTC
GCTTTTGGCATAAGTGGGCCCCCAGTGGGGGGCTCGCACGACGGGCTATTAGCTCAGTGGGTAGAGCGCGCC
CCTGATAATTGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATCG
GCGCCTGACCCTGAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTGTTTGAACCGGGGT
TTGAAACCAAACCTTCTCCTCAGGAGGATAGATGGGGCGATTGAGGTGAGATCCAATGTAGATCCAACCTTC
GATTCACCTCGTGGGATCCGGGCGGTCCGGGGGGGACCACCATGGCTCCTCTCTTCTCGAGAATCCATACAT
CCCTTATCAGTGTATGGACAGCTATCTCTCGAGCACAGGTTTAGGTTTCGGCCTCAATGGGAAAAATAAATG
GAGCACCTAACACGCATCTTCACAGACCAAGAACTACGAGATCACCCCTTTCATTCTGGGGTGACGGAGG
GATCATACCATTCGAGCCTTTTTTTTTTCATGCTTTTTCCCCGAGGTCTGGAGAAAGCTGAAATCAATAGGAT
TTCCCTAATCCTCCCTTACCGAAAGGAAGAGCGTGAAATCTTTTTCTTCCGACGGGACCAGGAGATTG
GATCTAGCCGTAAGAAGAATGCTTGGTATAAATACTACTTCTTGGTCTTCGACCCCGCAGTCACTACG
AACGCCCCGATCAGTGCAATGGGATGTGTCTATTTATCTATCTCTTGACTCGAAATGGGAGCAGGTTTGA
AAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGGTCTCTTAACGCCTCTTTTTTCTTCTCATCGGA
GTTATTTTACAAAGACTTGCCATGGTAAGGAAGAAGGGGGGAACAGGCACACTTGAGAGCGCAGTACAAC
GGAGAGTTGTATGCTGCGTTTCGGGAAGGATGAATCGTCCCGAAAAGGAATCTATTGATTCTCTCCCAATT
GGTTGGACCGTAGGTGCGATGATTTACTTCACGGGCGAGGTCTCTGGTTCAAGTCCAGGATGGCCAGCTG
CGCCAGGGAAAAAGAATAGAAGAAGCGTCTGACTCC [TTAATTAA] [TTGACAATTAATCATCCGGCTCGT]

PacI

trc promoter→ ←Primer P2

P3-P6 left border fragment consensus sequence

Primer P3→(aadA gene)

AAGTCAACATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGA
GAATGGCAGCGCAATGACATTCTTGAGGTATCTTCGAGCCAGCCAGATCGACATTGATCTGGCTATCTT
GCTGACAAAAGCAAGAGAACATAGCGTTGCTTGGTAGGTCCAGCGCGGAGGAACTCTTTGATCCGGTTC
CTGAACAGGATCTATTGAGGCGCTAAATGAAACCTTAACGCTATGGAACCTCGCCGCCCCGACTGGGCTGGC
GATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAA
GGATGTGCTGCGTGGCGACTGGGCAATGGAGCGCCTGCCGGCCAGTATCAGCCCGTCATACTTGAAGCTAGAC
AGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTAC

aadA stop/psbA 3'UTR→

GTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAGAGCGATCCTGGCCTAGTCTATAGGAGGT
TTTGAAAAGAAAGGAGCAGTAATCATTTTCTTGTCTATCAAGAGGGTGCTATTGCTCCTTTCTTTTTTTC
TTTTTATTTTACTAGTATTTTACTTACATAGACTTTTTTGTATTACATTATAGAAAAGAAGGAGAGGT
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AACTTGTTTCTCTTCTTGCTAATGTTACTATATCTTTTTGATTTTTTTTCCAAAAAAAATCAAATTTT
GACTTCTTCTTATCTCTTATCTTTGAATATCTTTATCTTTGAAATAATAATATCATTGAAATAAGAAAGA

AscI

trnA gene→

AGAGCTATATTTCGA [GGCGCGCC] CATGCATGCTCCACTTGGCTCGGGGGATATAGCTCAGTTGGTAGA
GCTCCGCTCTTGCAATTGGGTGCTTGGCATTACGGGTTGGATGTCTAATTGTCCAGGCGGTAATGATAGTA
TCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTAATGGGAAGAGGACCGAAACATGCCACTGAAAGA
CTCTACTGAGACAAAGATGGGCTGTCAAGAACGTCAAGAACGTAGAGGAGGTAGGATGGGCGAGTTGGTCA
ATCTAGTATGGATCGTACATGGACGGTAGTTGGAGTGGCGGCTCTCCTAGGGTTCCCTTATCGGGGATCC

CTGGGGAAGAGGATCAAGTTGGCCCTTGCGAACAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAA
ATGCGGCAAAAGGAAGGAAAATCCATGGACCGACCCCATCATCTCCACCCCGTAGGAACTACGAGATTACC
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GCTGTCCGCTCTCAGGTTGGGCAGTAAGGGTCGGAGAGGGCAATCACTCATTCTTAAACCAGCGTTCTT
AAGGCCAAAGAGTCGGCGGAAAAGGGGGGAAAGCTCTCCGTTCTGGTTTCCTGTAGCTGGATCCTCCGGA
ACCACAAGAATCCTTAGTTAGAATGGGATTCCAACCTCAGCACCTTTTGAGTGAGATTTTGAGAAGAGTTGC
TCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTTCGGGGGGGAGTTATTGTCTATCGTTGGCCT
CTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGTGGTTTACCCTGCGGCGGATGTCAGCGGTTTCGAGTC

trnA end

CGCTTATCTCCAACCTCGTGAACCTTAGCCGATACAAAGCTATATGACAGCACCAATTTTCCGATTGCGG

←Primer LCV2D = RB of LCV2

gttcgatctatgatttatcattcatggacgttgataagatccatccatttagcagcaccttaggatggcat

←Primer P6

agccttaaaattaagggcgag